Supporting Information

Minimizing Oxidation and Stable Nanoscale Dispersion Improves the Biocompatibility of Graphene in the Lung

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METHODS

1. Graphene Oxide Synthesis

1.1 Dispersed graphene production. Seven grams of natural graphite flakes (3061 grade material from Asbury Graphite Mills) were added to 70 mL of 2% w/v aqueous solution of Pluronic F 108NF (BASF Corporation) in a ~120 mL stainless steel beaker. This mixture was chilled in an ice water bath and ultrasonicated using a Fisher Scientific Model 500 Sonic Dismembrator using a 1/2" diameter tip for 2 hours at 60% amplitude (~65 W). The as-sonicated material was centrifuged in Beckman Coulter JS-7.5 rotor with a Beckman Coulter J26-XPI at 5000 rpm (~4,620g) for 30 min. The top 80% of the supernatant was retained for further processing. A step gradient was prepared by layering ~27 mL of the retained solution from the previous step on top of a dense 10.5 mL layer containing 60% w/v iodixanol and 2% w/v Pluronic F 108NF. These step gradients were ultracentrifuged in a SW 32Ti rotor (Beckman Coulter) for 24 hours at 32 krpm at a temperature of 22 °C. The band of highly concentrated graphene was isolated using a piston gradient fractionator (Biocomp Instruments). It was then diluted to 2.6 mg/mL, where the concentration was determined by using an average absorption coefficient for graphene of 2460 mL mg^{-1} m^{-1} at a wavelength of 660 nm[1]. This material was dialyzed in 20,000 molecular weight cut-off membranes (Pierce Slide-A-Lyzer) in a bath of 2% w/v Pluronic F 108NF for 3 days, with the bath solution being replaced daily. The solution was then diluted to a final concentration of 2 mg/mL for use as the well dispersed graphene sample.

1.2 Aggregated graphene production. Aggregated graphene was produced by flocculation of dispersed graphene via the addition of 4 parts isopropyl alcohol to one part dispersed graphene solution (by volume). The solution was then centrifuged in a Beckman Coulter JS-7.5 rotor with a Beckman Coulter J26-XPI at 5000 rpm (~4,620g) for 15 min to create a pellet of aggregated graphene. The supernatant was removed, replaced with DI

water, vortexed for 5 min, and finally underwent centrifugation as before. This wash step was repeated 4 times. The pellet was then mixed with deionized water to obtain a 2 mg/mL solution of aggregated graphene.

1.3 Graphene oxide (GO) production. GO was produced by a modified Hummer's method.[2] 115 mL of concentrated sulfuric acid (Mallinckrodt Baker) were cooled in a 2 L flask to 0 °C in an ice water bath. Subsequently, 5 g of natural graphite flakes (3061 grade material from Asbury Graphite Mills) were added to the cooled sulfuric acid. 15 g of $KMnO_4$ were gradually added to this mixture with stirring and cooling, so that the temperature did not reach 20 °C. The mixture was then heated to 35°C and stirred. This temperature was maintained for 2 hours under constant stirring, after which 230 mL of deionized water were added. After 15 minutes, the reaction was terminated by the addition of a 700 mL of deionized water. Following termination of the reaction, 12.5 mL of 30% H_2O_2 solution was slowly added. The mixture was then vacuum filtered and washed with 1.25 L of 1:10 HCl solution to remove metal ions, followed by further washing with 4 L of deionized water. The material was then suspended in 250 mL of deionized water. 12 mL of the solution from the previous step, diluted to ~4 mg/mL carbon, were sonicated in a 16 mL vial by a Fisher Scientific Model 500 Sonic Dismembrator with an 1/8" tapered horn tip for 1 hour at 20% amplitude (~10 W) while cooled in an ice water bath. The solution was then centrifuged in a tabletop centrifuge (Eppendorf Model 5424 Microcentrifuge with a FA-45-24-11 fixed-angle rotor) for 5 minutes at 15,000 krpm (~21,000 g) to remove any remaining large aggregates. The resulting solution was dialyzed in 20,000 molecular weight cut-off membranes (Pierce Slide-A-Lyzer) for 2 days in deionized water to remove any remaining acids or metal ions (the bath was replaced with fresh DI water several times). By slow evaporation of the liquid from a portion of the final solution, the optical absorbance at 330 nm was determined to be 3650 mL mg^{-1} m^{-1}. This value was used to dilute the final solution to 2 mg/mL for the final GO sample.

1.4 Optical characterization. Optical absorbance measurements were performed using a Varian Cary 5000 spectrophotometer. Background from the solution and cuvette were subtracted from the spectra. As the solutions were too concentrated to be characterized directly, the graphene and GO samples were diluted 40x prior to measurement in 2% w/v aqueous Pluronic F108NF or deionized water, respectively. Spectra were then equally scaled so the peak absorbance for graphene was 1. Raman spectra were obtained with a Renishaw inVia Raman microscope using an excitation wavelength of 514 nm. The spectra are an accumulation of 5 separate measurements after a linear background subtraction. As the Raman signal from the GO sample was almost 5x lower than that of the graphene, both spectra were normalized to a peak G band value of 1.

1.5 Atomic force microscopy sample preparation and measurements. Si wafers with a 100 nm thick oxide were immersed in a 2.5 mM (3-aminopropyl) triethoxysilane (APTES) aqueous solution for 30 min to functionalize them with a self-assembled monolayer. The wafers were then rinsed with water and dried with N_2. Graphene or GO solutions were then diluted to ~0.01 mg/mL with water, and a drop of this diluted solution was immediately placed on the wafer. After 10 minutes, the samples underwent two cycles of washing with water for ~5 sec and drying with N_2. To avoid issues with residual APTES, the GO sample underwent 30 min of heat treatment at 250 °C. Since this procedure was insufficient to remove excess Pluronic from the surface of the graphene sample, it was instead heat treated for one hour in air at 275 °C. AFM images were obtained via a Thermo Microscopes Autoprobe CP-Research AFM in tapping mode with conical probes (MikroMasch, NSC36/Cr-Au BS). Images were taken at several random locations on the sample and showed little variation. All images were obtained with the same tip and scanning conditions. Flakes with areas larger than 400 nm^2 were included in the analysis, as the occasional smaller features could not be definitively classified as flakes or residue. Additionally, a few features taller than 5 nm appeared to be large aggregates of flakes and were not included in this analysis.

1.6 X-ray photoelectron spectroscopy (XPS) sample preparation and measurements. For XPS measurements, films of graphene and GO were produced by vacuum filtration of 500 μL of 1 mg/mL graphene and GO solutions through Whatman Anodisc 20 nm pore size membranes. The graphene film was then washed with ~50 mL of deionized water to remove residual Pluronic. These samples were characterized using an Omicrometer ESCA probe (Omicrometer, Taunusstein, Germany) equipped with an EA125 energy analyzer (Keck Interdisciplinary Surface Science Center at Northwestern University). Photoemission was stimulated by a monochromated Al Kα beam radiating at 1486.6 eV. High resolution spectra of the C 1s region were acquired in steps of 50 meV. In order to minimize noise, 5 sweeps of this region were averaged, and a linear background was subtracted. The carbon-carbon bond peak was assigned a binding energy of 285.1 eV, and chemical shifts of +1.5, +2.5, and +4.0 eV were applied for hydroxyl, carbonyl, and carboxyl functional groups, respectively.[3] Graphene oxide was found to exhibit significantly increased levels of carbonyl and hydroxyl functional groups compared to graphene.

2. *In vivo* studies.

2.1. The administration of graphene preparations to the lungs of mice. The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. All mice were obtained from Charles River (Boston, MA). We anesthetized 8-12 week old, (20-25 g), male, C57BL/6 mice (Charles River, Wilmington, MA) with isoflurane and intubated them orally with a 20-gauge angiocath cut to a length that placed the tip above the carina. We then treated mice intratracheally with equal weights and volumes (50 μg/mouse in a total volume of 50 μL/mouse) of nanomaterials or control vehicles (water or Pluronic F 108NF) including (1) aggregated graphene (graphene in water), (2) dispersed graphene (graphene in 2% Pluronic) and (3) GO (in water) in two equal aliquots, 3 minutes apart.[4] Dispersed graphene and GO solutions were vortexed immediately prior to instillation. Aggregated graphene in water was vortexed and sonicated for 10 minutes prior

to instillation. After each aliquot the mice were placed in the right and then the left lateral decubitus position for 10-15 seconds. The mice used in this study weighed on average 25 g, while an average human weighs 70 kg. Based on weight, the equivalent human dose of graphene or graphene oxide used in this study would be 140 mg. The murine lung has a total lung capacity of ~1 ml while an average human total lung capacity is about 6000 ml. Based on volume, the equivalent human dose of graphene or graphene oxide used in this study would be 300 mg.

2.2. Histology. A 20-gauge angiocath was sutured into the trachea, the lungs and heart were removed *en bloc*, and the lungs were inflated to 15 cm H_2O with 4% paraformaldehyde. The heart and lungs were fixed in paraffin, and 5-μm sections were stained with hematoxylin/eosin and Masson's Trichrome stain (for detection of collagen fibers). Low power field images of whole mouse lungs were obtained using a Nikon microscope equipped with Neurolucida software.[4]

2. 3. Bronchoalveolar lavage (BAL) fluid. BAL fluid was obtained through a 20-gauge angiocath ligated into the trachea.[5] A 1.0-ml aliquot of PBS was instilled into the lungs and then carefully aspirated three times. A 200-μl aliquot of the BAL fluid was placed in a cytospin and centrifuged at 500*g* for 5 min. The glass slides were Wright stained and subjected to a blinded manual cell count and differential. The remaining BAL fluid was centrifuged at 200*g* for 5 min, and the supernatant was used for the measurement of BAL protein (Bradford).

2.4. Measurement of plasma thrombin antithrombin complex (TAT) levels. Venous blood was dicollected into sodium citrate (0.32% final dilution) containing syringe via direct right atrial puncture of mice 24 hours after intratracheal treatment with control vehicles (water or 2% Pluronic F 108NF) or nanomaterials (aggregated

graphene, dispersed graphene and GO). Plasma was separated by centrifugation at 1,500*g* for 15 minutes and plasma TAT complex levels were measured using Enzygnost TAT Micro kit (Dade Behring Inc.).[5]

2.5. Measurement of total lung collagen. Total collagen was in homogenized lung was measured using a picosirius red assay as previously described.[6] The lungs were harvested and suspended in 0.5 N acetic acid and then homogenized first with a tissue homogenizer (30 seconds on ice) and then using 12 strokes in a Dounce homogenizer (on ice). The resulting homogenate was spun (>10,000 X g) for 10 minutes and the supernatant was used for subsequent analysis. Collagen standards were prepared in 0.5N acetic acid using rat tail collagen (Sigma-Aldrich). Picrosirius red dye was prepared by mixing 0.2 g of Sirius Red F3B (Sigma-Aldrich) with 200 ml of saturated picric acid in water (solid picric acid maintained at the bottom of the flask to insure saturation). One ml of the Picrosirius red dye was added to 50μL of the collagen standard or the lung homogenates and they were mixed continuously at room temperature on an orbital shaker for 30 minutes. The precipitated collagen was then pelleted and washed once with 0.5 N acetic acid (>10,000 x g, 10 minutes). The resulting pellet was resuspended in 500 μL of 0.5 M NaOH and Sirius red staining was quantified spectrophotometrically (540 nm) using a colorometric plate reader (BioRad).

2.6. Electron Microscopy. To prepare samples for electron microscopy, lung tissue was immersed and fixed in 2.5% glutaraldehyde in 0.1M Sodium Cacodylate buffer, buffer rinsed, and post fixed in 2% Osmium tetroxide. After fixation, samples were washed in cacodylate buffer, enblocked stained with 3% Uranyl acetate, washed with distilled water, dehydrated in ascending grades of ethanol using propylene oxide as transitional fluid for resin infiltration, and embedded in EMbed 812 and Araldite 502 mixture. Images were acquired on a Tecnai Spirit TEM.[4]

3. *In vitro* studies.

3.1. Measurement of mitochondrial reactive oxygen species (ROS). Mitochondrial ROS were measured in mouse alveolar macrophages (MHS) and epithelial cells (MLE 12) (catalog no CRL-2019 and CRL-2110, respectively, ATCC, Manassas, VA). We employed a mitochondrially localized oxidant sensitive GFP probe that was originally described by Hanson and colleagues who validated its responsiveness to a variety of intracellular oxidants both ex vivo and in living cells.[7-8] We have previously described the procedure for generating a lentiviral vector encoding this probe and confirmed mitochondrial localization of the expressed protein.[9] After treatment, the cells were removed from the plate using trypsin, and equal aliquots of the resulting suspension were transferred to tubes containing media alone or media containing 1mM DTT or 1mM t-butyl hydroperoxide (TBHP). After 10 minutes, the ratio of fluorescence (emission of 535 nm) at excitations of 400 and 490 nm was measured in 5,000 cells per condition using a DakoCytomation CyAn high speed multilaser droplet cell sorter. The oxidation state of the cells was calculated as the completely reduced ratio (DTT) less the untreated value divided by the difference in the ratio observed with DTT and TBHP.

3.2. Measurement of apoptosis. Apoptosis was measured using a commercially available photometric immunoassay that detects histone-associated DNA fragments (Roche Applied Science). [9]

3.3. Measurement of oxygen consumption. MHS cells were grown on a 96 well plate, and 24 hours later oxygen consumption was measured using a Seahorse XF analyzer as we have previously described.[10]

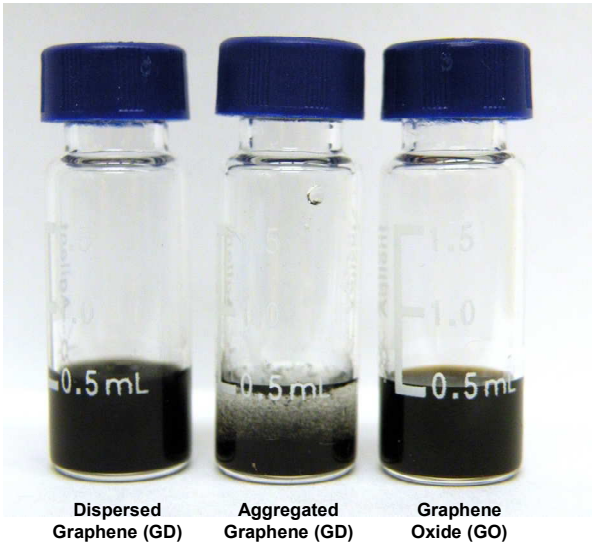

Figure S1. Photographs of the different preparations of graphene. Graphene preparations were produced as described and aliquots were placed in glass tubes and photographed (Pristine graphene dispersed in 2% Pluronic (GD) (Left), Pristine graphene dispersed in PBS (GA) (Center) and graphene oxide in PBS (GO) (Right)). The pristine graphene in PBS was vortexed immediately prior to being photographed. The other preparations have been stable as shown for > 6 months.

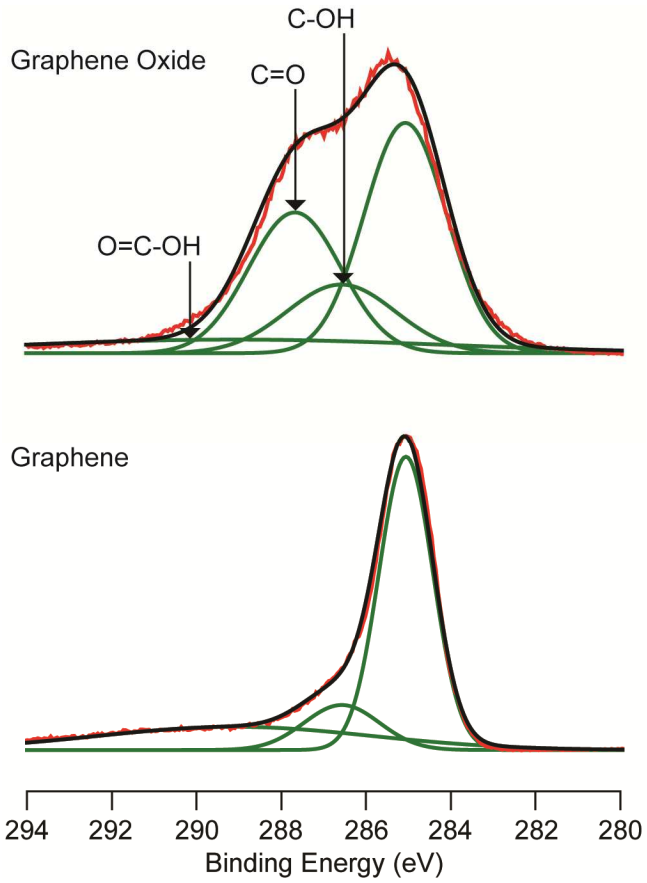

Figure S2. XPS spectra of graphene oxide and Pluronic dispersed graphene in the carbon 1s region. XPS spectra after linear background subtraction are shown in red, the fits to XPS spectra in black, and individual peaks in green.

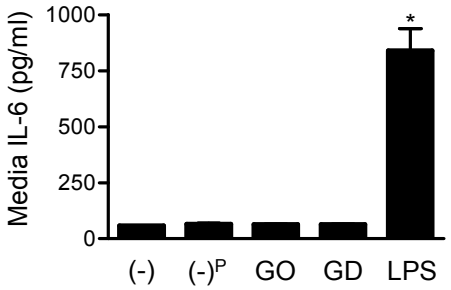

Figure S3. The administration of GO or GD to alveolar macrophages does not directly induce the release of IL-6. MHS cells were treated with PBS (-), 2% Pluronic in PBS $(-)^P$, graphene oxide (GO) (10 μg/cm^2) or dispersed graphene (GD) (10 μg/cm^2) or lipopolysaccharide (LPS) (10 ng/ml) and the levels of IL-6 were measured in the media 24 hours later. * $P < 0.05$ compared with control. N = 3 for all measures.

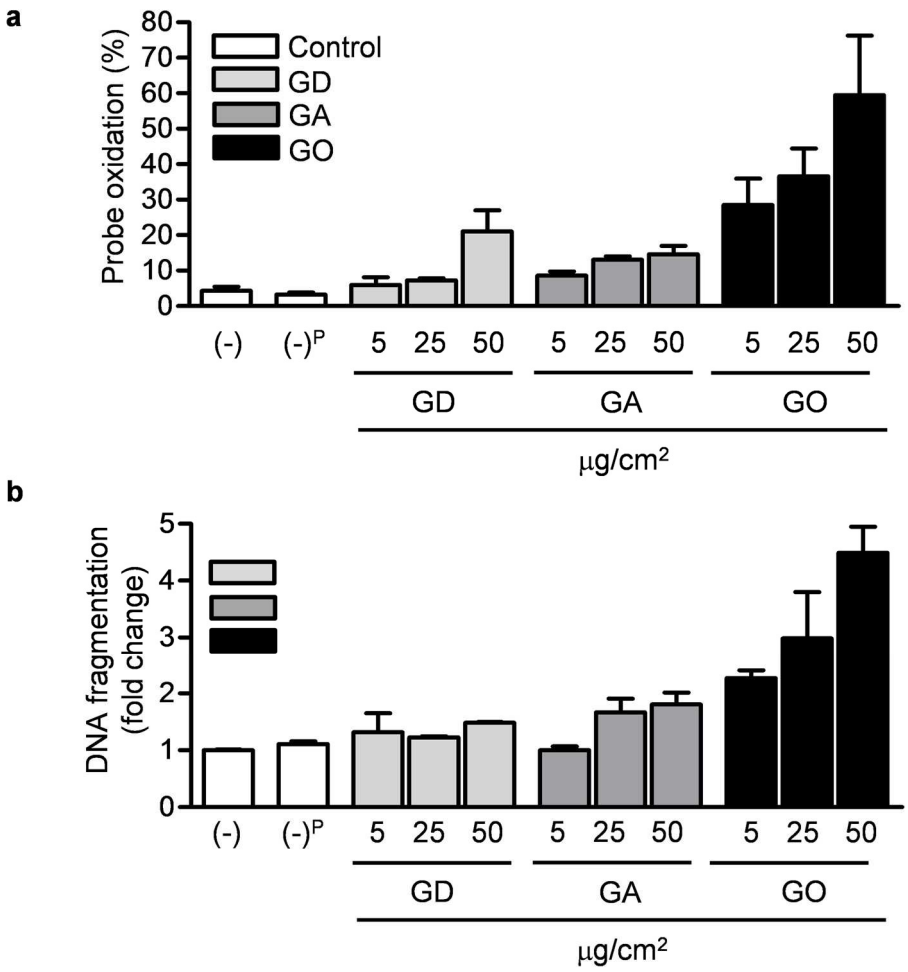

Figure S4. Graphene oxide, but not graphene, induces the mitochondrial generation of ROS and cell death in an alveolar epithelial cell line. (a) Cells from a murine alveolar epithelial cells line (MLE-12) stably expressing a mitochondrially localized oxidant sensitive GFP probe (Mito-Ro-GFP) were exposed to increasing concentrations of dispersed graphene in 2% Pluronic F 108NF (GD), aggregates of graphene in water (GA), graphene oxide (GO), water (-) or water with 2% Pluronic $(-)^P$ and oxidation of the probe was measured using flow cytometry 4 hours later. (b) MLE-12 cells were exposed as in (a) and cell death was measured 24 hours later using a DNA fragmentation ELISA.

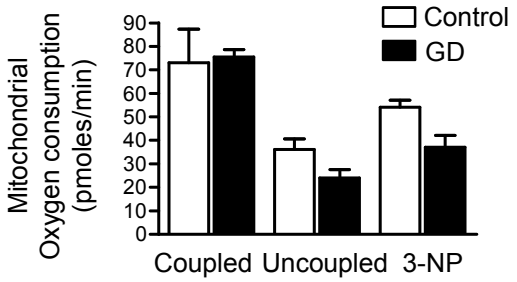

Figure S5. Oxygen consumption was measured in MHS cells treated with vehicle (2% Pluronic) or dispersed graphene (GD) using a Seahorse XF analyzer. Coupled (oligomycin sensitive) and uncoupled (oligomycin insensitive) mitochondrial (rotenone/antimycin A inhibitable) respiration are shown. In addition, the cells were treated with 3-NP (2 mM) and oxygen consumption was measured. P=NS for comparisons between vehicle and GD treated cells.

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